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(54) Title: POLYNUCLEOTIDES AND POLYPEPTIDES OF AVIAN LEPTIN-RECEPTOR

(57) Abstract: Polynucleotide and polypeptide sequences of avian leptin-receptors and uses thereof.

POLYNUCLEOTIDES AND POLYPEPTIDES OF AVIAN LEPTIN-RECEPTOR

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to polynucleotides encoding leptin-receptors and to polypeptides encoded by these polynucleotides and, more particularly, to polynucleotides encoding avian leptin-receptors and to polypeptides encoded by these polynucleotides.

Leptins and leptin-receptors in mammals:

The satiety hormone leptin and leptin-receptor (LR) are key regulators in the control of food intake, glucose metabolism, fat metabolism, energy expenditure, puberty, fertility, and immunity in mammals (reviewed in, Campfield *et al.*, 1996; Tartaglia, 1997; Auwerx and Steels, 1998; Freidman and Halaas, 1998).

The circulating hormone leptin is produced primarily in the adipose tissues and signals to the hypothalamus indications relating to energy reserves. The gene encoding leptin was identified by positional cloning in 1994 (Zhang *et al.*, 1994) in a mouse homozygote for a Mendelian segregating recessive mutation (*ob*) resulting, in its homozygous state (*ob/ob*), in a profound obese phenotype, including three times higher weigh than normal mice, five fold increase in body fat content, diabetes, infertility, low body temperature, low activity and hampered immunity.

The mouse and human leptin-receptor genes were discovered in 1995 (Tartaglia, *et al*, 1995; Luoh *et al.*, 1997, respectively) and led to the cloning of the leptin-receptor gene (or partial sequences thereof) in other mammalian species, including, rat, pig, sheep and cow. The mammalian leptin-receptor mediates the weight regulatory effects of its ligand, leptin, through interactions with a cytoplasmic kinase.

It was thereafter found that exogenous administration of recombinant leptin to *ob/ob* mice leads to a complete normalization of all aspects of the

ob/ob phenotype (see, for example, Ahima *et al.*, 1997; Halaas *et al.*, 1997; Kamohara *et al.*, 1997; and Seeley *et al.*, 1996). The expression and secretion of leptin in mammals was shown to be highly correlated with body fat mass and adipocyte size.

At least six alternatively spliced leptin-receptor transcripts were identified in mammals designated Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, and Ob-Re (Tartglia *et al.*, 1996; Lee *et al.*, 1998). Among these forms of the receptor, only the long variant (Ob-Rb) contains the cytoplasmic domain that interacts with the Jak kinase (JAK), and the signal transducer and activator of transcription (STAT) proteins, which have been implicated with signal transduction of the leptin-receptor pathway (White *et al.*, 1997).

OB-Rb is expressed primarily in the paraventricular (VPN), ventromedial (VMH), dorsomedial (DMH), lateral (LN) and arcuate neurons of the hypothalamus - regions known to be associated with body weight regulation (Hetherington and Ranson 1942; Mercer *et al.*, 1996; Fei *et al.*, 1997).

In the obese diabetic (*db/db*) mouse, a mutation is found in the OB-Rb form of the receptor, leading to a phenotype of extreme obesity and infertility, nearly indistinguishable from the phenotype of *ob/ob* mice.

These obesity mutations (*db* and *ob*) demonstrate a very unique situation in which a single protein and its related receptor play a key role in the two most agriculturally important parameters - feeding and fertility.

Implications to poultry industry:

While evidence for the therapeutic value of leptin and leptin-receptor in humans accumulate (Friedman and Halaas, 1998), it still remains unclear whether this unique control mechanism is conserved in non-mammalian vertebrates, such as avians, as well.

Chicken, turkey and other poultry are the main source of protein for human consumption and as such, commercial poultry live-stocks, have been genetically selected for fast growth.

As a result of such selection, broilers, for example, tend to over eat, which results in wastage of food and undesired accumulation of fat. Among broiler breeders, the problem of over eating, fat deposition and loss of fertility imposes a need to restrict feed intake by artificial means.

The situation is quite different for farm turkeys which accumulate insufficient amounts of fat in their carcass. It is believed that increasing the appetite of turkeys could serve both to improve fat deposition and to enhance growth rates.

In geese, for example, the need of force-feeding for the production of high quality *fois-gras* involves highly qualified manpower and evokes public objection, which is the biggest threat to this branch of the poultry industry in some areas of the Globe.

The ostrich branch of poultry is much less developed, yet seems to be of high future commercial potential.

Many of the problems listed hereinabove with respect to poultry breeding could be improved by controlling food consumption, energy expenditure and the like. Future tools to enhance puberty and improve fertility are important for growers of laying hens, turkeys, ostriches, gees and other commercially grown poultry.

Some indirect experimental results suggest similarities between the mechanisms regulating appetite and other aspects of energy balance among mammals and avians, no direct evidence has so far been provided in this respect (Lepkovsky and Yasuda, 1966; Snapir *et al.*, 1973; Kuenzel and Helms, 1967, Kuenzel *et al.*, 1987; Richardson *et al.*, 1995, Kuenzel and McMurty, 1998, Fraley and Kuenzel, 1993).

There is thus a widely recognized need for, and it would be highly advantageous to know whether (i) avians posses a mechanism for regulating appetite and other aspects of energy balance, such as, but not limited to, food consumption, energy expenditure, puberty, fertility and immunity, which is similar to that found in mammals, and if so, (ii) to clone the genes

encoding the protein associated with such a mechanism; and (iii) to use such cloned genes to control appetite and other aspects of energy balance in avians, commercially bred poultry in particular.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a nucleic acid sequence encoding an avian, poultry in particular, leptin-receptor.

It is a further object of the present invention to provide a polynucleotide encoded by the avian leptin-receptor nucleic acid.

It is a further object of the present invention to provide an assay which will enable screening for leptin agonists and antagonists.

It is a further object of the present invention to provide for the ability to attenuate leptin activity in vivo, to thereby manipulate food intake, fat deposition, puberty and fertility in poultry.

It is a further object of the present invention to use the nucleic acid and polypeptide according to the present invention to isolate an avian leptin gene.

While reducing the present invention to practice, the chicken leptin-receptor gene was cloned, analyzed and sequenced. Cloning the chicken leptin-receptor gene represents the first non-mammalian derived isolated leptin-receptor gene.

Thus, according to one aspect of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence at least 70 % identical with a nucleic acid encoding a polypeptide as set forth in SEQ ID NO:28 or a portion thereof longer than 30 contiguous amino acids, as determined using the Smith-Waterman algorithm and the following parameters: Matrix = Blosum62, gapopen = 10.0 and gapextend = 0.5.

According to another aspect of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence hybridizable with a nucleic acid encoding a polypeptide as set forth in SEQ

ID NO:28 under a hybridization selected from the group consisting of stringent hybridization and moderate hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC, 5 x Denhardt's solution, 1 % SDS and 100 μg/ml denatured salmon sperm DNA, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final at 60 °C, whereas moderate hybridization is effected by a hybridization solution of 6 x SSC, 5 x Denhardt's solution, 1 % SDS and 100 μg/ml denatured salmon sperm DNA, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C.

According to further features in preferred embodiments of the invention described below, the nucleic acid sequence is as set forth in SEQ ID NO:27 or a portion thereof longer than 90 contiguous nucleotides and natural and man induced variants thereof.

According to still further features in the described preferred embodiments the nucleic acid sequence is of an avian.

According to still further features in the described preferred embodiments the polypeptide is capable of binding leptin under physiological conditions.

According to still further features in the described preferred embodiments the nucleic acid sequence is as set forth in SEQ ID NO:27.

According to still further features in the described preferred embodiments the isolated polynucleotide further comprising a vector ligated to the nucleic acid sequence.

According to still further features in the described preferred embodiments the nucleic acid sequence is a product of a polymerase chain reaction.

According to yet another aspect of the present invention there is provided a recombinant protein comprising an amino acid sequence at least 70, at least 80, at least 90 or at least 90-100 % homologous (identical +

similar amino acids) with SEQ ID NO:28 or a portion thereof longer than 30 contiguous amino acids, as determined using the Smith-Waterman algorithm and the following parameters: Matrix = Blosum62, gapopen = 10.0 and gapextend = 0.5.

According to further features in preferred embodiments of the invention described below, the amino acid sequence is as set forth in SEQ ID NO:28 and natural and man induced variants thereof.

According to still further features in the described preferred embodiments the amino acid sequence is of an avian.

According to still further features in the described preferred embodiments the polypeptide is capable of binding leptin under physiological conditions.

According to still further features in the described preferred embodiments the amino acid sequence is as set forth in SEQ ID NO:28.

According to still another aspect of the present invention there is provided a single stranded polynucleotide of at least 18 nucleotides hybridizing with a nucleic acid sequence as set forth in SEQ ID NO:27 under a hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 g/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 1 - 1.5 °C below the T_m, final wash solution of 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS at 1 - 1.5 °C below the T_m; moderate hybridization is effected by a hybridization solution of 6 x SSC and 0.1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 µg/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 2 - 2.5 °C below the

T_m, final wash solution of 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS at 1 - 1.5 °C below the T_m, final wash solution of 6 x SSC, and final wash at 22 °C; whereas mild hybridization is effected by a hybridization solution of a hybridization solution of 6 x SSC and 1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 µg/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 37 °C, final wash solution of 6 x SSC and final wash at 22 °C.

According to an additional aspect of the present invention there is provided a single stranded polynucleotide of at least 18 contiguous nucleotides of SEQ ID NO:27.

According to yet an additional aspect of the present invention there is provided a genetically modified cell expressing the polynucleotide described herein in a sense or antisense orientation.

The present invention successfully addresses the shortcomings of the presently known configurations by providing avian leptin-receptor sequences which are shown herein to share little homology with corresponding mammalian genes and which may therefore be employed to use such cloned genes to control the appetite and other aspects of energy balance in avians, commercially bred poultry in particular. Additional objects and advantages of the present inventions will become apparent reading the description of the preferred embodiments that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIGs. 1a-b show steps of a Southern blot analysis of chicken leptin-receptor phage 1. 1a - restriction enzyme digestion and separation on 1 % agarose gel stained with ethidium bromide. 1b - blotting and hybridization with a human leptin-receptor probe. Washings were with 0.5 x SSC/0.1 % SDS at 42 °C. Exposure was for 3 days. The 1.4 kb Eco RI-Pvu II and the 7 kb Eco RI fragments were subcloned and further analyzed.

FIG. 2 shows the nucleotide sequence of the Eco RI-PVU II fragment derived from phage 1 and a comparison thereof to human leptin-receptor exons 9 and 10. This fragment was subcloned in Blue-Script (BS) and sequenced with primers from the BS cloning polylinker. The upper sequence is the chicken leptin-receptor sequence, whereas the lower sequence is the human leptin-receptor exons 9 and 10 sequence. Exonal sequences are shown in bold face.

FIG. 3 shows RT-PCR amplification of chicken leptin-receptor cDNA fragments. Samples of mRNA were prepared from female Leghorn chicken hypothalamus and fat. First-strand cDNA was prepared using random hexamer primers. Exon 9 (forward) and exon 15 (reverse) derived primers served for PCR amplification. The resulting PCR products were electrophoresed on a 1 % agarose gel, blotted onto a nitrocellulose filter, hybridized with a chicken derived exons 9 and 10 leptin-receptor probe, washed and autoradiographed for 4 hours with Kodak BioMax MR X-ray film at -70 °C.

FIGs. 4a-b show steps of a Northern blot analysis of chicken leptin-receptor in the hypothalamus. 4a - Hybridization was performed with a chicken derived leptin-receptor probe under stringent wash conditions. Autoradiography was for 4 days with Kodak BioMax MR X-ray film at -70

°C. 4b - Ethidiume bromide staining of the RNA indicates the integrity of the RNA and provides for ribosomal RNA (28S and 18S) size reference.

FIG. 5 shows PCR amplification results of DNA derived from a chicken adipose tissue derived cDNA library. DNA was extracted from a sample of the cDNA library and served as template for PCR amplifications in which primers designed according to the so far characterized chicken leptin-receptor exons 10 and 15 and the phage polylinker sequence (T7 and T3) were employed. An example of amplification to the 3' end is shown in the Figure. The first PCR was performed with primers from the phage cloning polylinker (either T3 or T7) and with a chicken leptin-receptor exons 10 forward primer (10F), with touchdown cycles from 65 to 55 °C. Nested PCR was then performed with chicken leptin-receptor 15 forward primer (15F). PCR products were electrophoresed on 1 % agarose gel and were stained with ethidium bromide and photographed under UV illumination. A negative image is shown.

FIG. 6 shows amplification of cDNA ends using the Marathon RACE kit from Clontech. First (1st) and second (2nd) amplifications were performed according to the kit's instruction protocol.

FIGs. 7 shows amplification of chicken leptin-receptor sequences from DNA of chicken leptin-receptor genomic phages 1 and 2. Amplification to the 5' end with T7 T3 phage primers and chicken leptin-receptor exon 10 reverse primer is shown for two independent clones (phage 1 and 2).

FIG. 8 is a schematic presentation of the two genomic phages and the approach which eventually led to the identification and isolation of exons 6-20 of the chicken leptin-receptor gene.

FIG. 9a provides the nucleotide sequence of the coding region (exons 3-20) of the chicken leptin-receptor gene according to the present invention.

FIG. 9b provides the translation of exons 3-20 of the chicken leptin-receptor gene.

FIG. 10 is a schematic presentation four phages shown to include chicken leptin-receptor genomic DNA sequences. The first two (phages 1 and 2) has already been described in Figure 8 above. Phages 3 and 4 were identified in an additional screen of a chicken genomic DNA library.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of polynucleotide and polynucleotide sequences of avian leptin-receptor which can be used, in a variety of ways, to study, modulate, select for, and control appetite and other aspects associated with energy balance in avians. Specifically, the present invention can be used to provide an assay which will enable screening for leptin agonists and antagonists; to provide for the ability to attenuate leptin activity in vivo, to thereby manipulate food intake, fat deposition, puberty and fertility in poultry; and to isolate an avian leptin gene.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

While reducing the present invention to practice, the isolation of a chicken leptin-receptor gene was attempted. As further detailed hereinunder, albeit the knowledge derived from mammalian leptin-receptor genes, this task has proven difficult and non-trivial. In retrospect it can be concluded that this was due to a number of reasons, including, but not

limited to, (i) the length and number of introns spacing the 20 exons of the gene; (ii) the very low representation of chicken leptin-receptor mRNA in chicken tissues; and therefore (iii) the low (or no) representation of cDNA sequences corresponding to chicken leptin-receptor in an adipose tissue derived cDNA library; (iv) the low homology with mammalian leptin-receptor genes; and (v) the difficulty to PCR amplify chicken leptin-receptor derived sequences, which, in most cases required nested amplification and resulted in non-specific products.

Thus, the following path was taken while reducing the present invention to practice. A human leptin-receptor probe corresponding to exons 9-10, which include regions conserved among other mammalian leptin-receptor genes was prepared. This probe served to screen an adipose tissue derived chicken cDNA library, but no positive clones were identified even under the mildest hybridization conditions, although fat tissue is known to express the leptin-receptor in mammals and could be readily detected. The same probe was then used to screen a chicken genomic library and several genomic clones were identified and subsequently isolated and analyzed to reveal chicken leptin-receptor exons 9 and 10. These exons, which are expected to be most conserved, showed an average homology of 75 % to the corresponding human derived sequences. Retrospectively it was found out that this region shows the highest homology between chicken leptin-receptor and corresponding mammalian leptin-receptors.

Since the leptin-receptor gene in mammals is estimated to be about 100 kb long, further efforts were directed, at first, at isolating cDNA sequences of the chicken leptin-receptor gene. Thus, a chicken exon 9 and 10 leptin-receptor probe was used to re-screen the cDNA library, yet, again this effort did not result in isolation of positive cDNA clones. Then, the use of a longer probe was attempted. Subcloning and sequencing of a larger fragment from the genomic clone isolate, and comparing the sequence

thereof to the GeneBank database led to the identification of chicken leptin-receptor exon 15. Using RT-PCR with a reverse primer from exon 15 and a forward primer from exon 9 yielded a 1 kb cDNA fragment. This longer fragment was used to screen the cDNA library, yet again this screening effort did not result in isolation of positive cDNA clones. At this point is was assumed that the cDNA library is poor in chicken leptin-receptor derived sequences due to low level of expression thereof in chicken adipose cells. This assumption is reasonable based on the fact that (i) the cDNA library employed is known to be highly representative; and (ii) that while attempting to RT-PCR amplify chicken leptin-receptor sequences, the use of nested amplification was always required. Further attempts to use several rapid amplification of cDNA ends (RACE) procedures and to fish mRNA sequences using biotinylated oligonucleotides, as further described in the Examples section that follows, all failed, reassuring the above assumption that the level of expression of leptin-receptor mRNA in chicken adipose cells is low.

At this point it was decided to take a new approach. The new approach was to isolate the extreme coding exons of the chicken leptin-receptor gene and to employ RT-PCR to then bridge over the missing exons. To this end, probes derived from the mouse leptin-receptor gene exons 3 and 20, which are the extreme coding exons, were prepared by RT-PCR and the genomic library was screened once more using these probes, however, no positive clones were isolated. Indeed, following the isolation and sequencing of exon 20 of the chicken leptin-receptor, it was found that the level of homology between the chicken and the mouse sequences is too low to sustain hybridization.

The bridging concept was then applied to large fragments of DNA which were amplified by PCR from DNA derived from the genomic isolates using leptin-receptor primers in combination with primers hybridizing with the library vector (T3 and T7). Due to the expected length of the amplified

segments, amplification was attempted, at first, employing high-fidelity enzymes for long template PCR, however, no positive PCR products were obtained. Surprisingly, while attempting the same experimental protocol with a conventional enzyme, not at all recommended for long template PCR, specific positive bands were obtained, which enabled the identification of exons 6 and 20 of the chicken leptin-receptor gene. RT-PCR bridging using exon 6 and exon 20 primers enabled to obtain a cDNA spanning exons 6-20 of the chicken leptin-receptor gene, which cDNA was subsequently cloned and sequenced. Encouraged by the success of this approach, additional genomic clones extending to the 5' region of the chicken leptin-receptor gene were isolated–for the characterization of the missing coding exons 3-5. Purified phage DNA was digested with EcoRI enzyme and the resulting fragments were subcloned in pGM-T vector (Promega). Sequencing 15 independently isolated subclones led to the identification of exons 5 and 4. Exon 3 was dificult to identify because of its small size and low sequence similarity. Therefore the RACE procedure was performed again using the Marathon kit (Clontech), this time employing an exon 6 reverse primer (6RII) and an exon 4 reverse nested primer (4R). Template RNA (0.6 μg polyA+) was prepared from 20 White-Leghorn chickens chicken choroid plexi. RACE products were subcloned into the pGM-T vector and sequencing of 5 independently isolated subclones confirmed the sequence of exon 3. Conformation was done in addition by bridging-PCR using exon 3 primer and exon 10 primer. Thereby a full-length chicken leptin-receptor cDNA, exon 3 to 20 was obtained.

Thus, while reducing the present invention to practice, the full length chicken leptin-receptor gene was cloned, analyzed and sequenced. Cloning the chicken leptin-receptor gene represents the first non-mammalian derived isolated leptin-receptor gene. It is shown herein that this newly isolated gene shares about 60 % homology at the nucleotides level with

corresponding mammalian genes, whereas a much higher level of homology characterizes these mammalian genes among themselves.

According to one aspect of the present invention there is provided an isolated polynucleotide. The isolated polynucleotide according to the present invention includes a nucleic acid sequence at least 70 % identical, preferably, at least 80 % identical, preferably, at least 85 % identical, more preferably, at least 90 % identical, most preferably 90-100 % identical with a nucleic acid encoding a polypeptide as set forth in SEQ ID NO:28 or a portion thereof longer than 30, preferably longer than 50, more preferably longer than 70, more preferably longer than 100, yet more preferably, longer than 150 contiguous amino acids, as determined using the Smith-Waterman algorithm and the following parameters: Matrix = Blosum62, gapopen = 10.0 and gapextend = 0.5.

The above specified degree of sequence identity is expected to include leptin-receptor sequences derived from avians and reptiles which are evolutionary close, yet, and as further shown in Table 2 in the Examples section that follows, mammalian leptin-receptor genes share only about 60 % nucleotide sequence identity with the chicken gene.

According to another aspect of the present invention there is provided an isolated polynucleotide which includes a nucleic acid sequence of at least 18, preferably at least 25, more preferably at least 50 nucleotides long, hybridizable with a nucleic acid encoding a polypeptide as set forth in SEQ ID NO:28 under a hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization.

According to another aspect of the present invention there is provided a single stranded polynucleotide of at least 18, preferably at least 25, more preferably at least 30, 40, 50, 60, 70 or at least 80 nucleotides hybridizing with a nucleic acid sequence as set forth in SEQ ID NO:27 under a hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization.

According to the present invention, hybridization for long nucleic acids (above 200 bp in length) is effected by stringent or moderate hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC, 5 x Denhardt's solution, 1 % SDS and 100 g/ml denatured salmon sperm DNA, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, whereas moderate hybridization is effected by a hybridization solution of 6 x SSC, 5 x Denhardt's solution, 1 % SDS and 100 g/ml denatured salmon sperm DNA, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C.

Further according to the present invention, hybridization for shorter nucleic acids (below 200 bp in length, e.g. 18-40 bp in length) is effected by stringent, moderate or mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 g/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 1 - 1.5 °C below the T_m, final wash solution of 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS at 1 - 1.5 °C below the T_m; moderate hybridization is effected by a hybridization solution of 6 x SSC and 0.1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 g/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 2 - 2.5 °C below the T_m, final wash solution of 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS at 1 - 1.5 °C below the T_m, final wash solution of 6 x SSC, and final wash at 22 °C; whereas mild hybridization is effected by a hybridization solution of a hybridization solution of 6 x SSC and 1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 μg/ml denatured salmon sperm DNA and 0.1 % nonfat dried

milk, hybridization temperature of 37 °C, final wash solution of 6 x SSC and final wash at 22 °C.

The above specified hybridization conditions, the stringent hybridization conditions in particular, allow detectable hybridization between avian and reptile derived leptin-receptor sequences, yet does not allow hybridization between these sequences and mammalian derived leptin-receptor sequences.

According to a preferred embodiment of the present invention, the nucleic acid sequence is as set forth in SEQ ID NO:27, or a portion thereof longer than 90, 100, 150, 200 or preferably 300 contiguous nucleotides and natural (e.g., allelic and non-allelic) and man induced variants (e.g., mutations and innocuous or substantially innocuous alterations) thereof.

The polypeptide encoded by the polynucleotide of the present invention is preferably capable of binding leptin under physiological conditions. In other words, according to preferred embodiments of the present invention, the polypeptide encoded by the polynucleotide of the present invention includes a leptin binding site. In mammals the leptin binding site of the leptin-receptor is encoded by exons 8-13.

According to another preferred embodiment of the present invention the isolated polynucleotide further includes a vector ligated to the nucleic acid sequence herein described. Such a vector can be a phage vector, a viral vector, a plasmid, a cosmid, a phagemid, an artificial chromosome and the like and is characterized in that it can propagate within cells. The vector is preferably an expression vector from which sense and antisense sequences are expressible to thereby up or down regulate the level of leptin-receptor mRNA levels in avians. In this case, the vector includes expression control elements, such as, but not limited to, a promoter and one or more enhancers. Such expression control elements can be tissue specific, so as to direct tissue specific regulation of leptin-receptor mRNA levels. These vectors can be used to genetically modify cells to thereby obtain a genetically

modified cell expressing the polynucleotide described herein in a sense or antisense orientation. Alternatively, the vector can be of a type suitable for gene knock-out, gene knock-in or for overexpression in transgenic animals. In this case the vector preferably includes both positive and negative selection markers, as well known in the art. The vector is preferably integrateable into the genome of the host.

The polynucleotide according to the present invention can be a product of a polymerase chain reaction or any other reaction used for amplification of nucleic acid sequences, such as strand displacement amplification and the like. Alternatively, it can be a product derived from a clone by, for example, digestion with restriction endonucleases.

According to yet another aspect of the present invention there is provided a recombinant leptin-receptor protein. The protein according to the present invention includes an amino acid sequence at least 70 %, 75 %, 80 %, 88 %, or preferably at least 90 % or at least 95 % identical with SEQ ID NO:28 or a portion thereof longer than 30, 40, 50, 70, 90, 150, 200, 300, 500, 700 or preferably longer than 1000 contiguous amino acids of SEQ ID NO:28, as determined using the Smith-Waterman algorithm and the following parameters: Matrix = Blosum62, gapopen = 10.0 and gapextend = 0.5.

Thus, the recombinant proteins herein described differ from mammalian leptin-receptor proteins which exhibit only about 50 % identity with SEQ ID NO:28 (see Table 1 in the Examples section that follows).

According to a preferred embodiment of the present invention the amino acid sequence is as set forth in SEQ ID NO:28 and natural and man induced variants thereof.

The protein according to the present invention can be used for the manufacture of anti-avian leptin-receptor antibodies, both poly and monoclonal antibodies by means well known to the skilled artisan.

A soluble form of the protein according to the present invention can be used as a drug to control leptin activity because administration of such a soluble form to the blood system of avians will result in capture of circulating leptin therefrom. It will be appreciated in this respect that in mammals, one of the alternatively spliced variants of the leptin-receptor, OBR-e, encodes a soluble form of the receptor, which lacks the intracellular and the trans-membrane domains (Fei H *et al.*, 1997; Lollmann *et al.*, 1997). In an *in vitro* system, the soluble receptor blocks binding of leptin to the longer form of the membranal leptin-receptor (Liu *et al.*, 1997), suggesting that the soluble receptor could normally act as an antagonist of leptin activity. The soluble form can be administered as a purified protein or small peptide derivatives thereof, or alternatively it can be introduced by genetic engineering (gene therapy) techniques by providing avians (e.g., transgenic avians) including cells which have been genetically modified to produce and secrete the soluble form of the receptor. In both cases possible effects could involve food intake, thermoregulation, fat deposition, puberty, fertility, immunity, angiogenesis and the like.

In addition, leptin-receptor cDNA according to the present invention can be used to establish a bioassay for chicken leptin activity in cultured cells. Ba/F3 cells are murine hematopoietic cells which dependent on IL-3 for survival and proliferation. However, expression of the long form of the leptin-receptor therein confers Ba/F3 cells IL-3 independent, provided that the growing cells are subjected to leptin, e.g., human leptin (see, for example, Gainsford *et al.*, 1996). Such genetically modified cells can be used in an assay for monitoring mammalian leptin levels in biological samples. This assay system is expected to be much less or not responsive at al to avian leptins. Expressing the polynucleotide according to the present invention in the Ba/F3 cell line is expected to render these cells IL-3 independent in the presence of avian leptin. Such a genetically modified cell line can therefore be used to evaluate biological activity of chicken

leptin and to screen libraries of potential leptin agonists and antagonists. It will be appreciated that other cells lines or yeast cells which depend on serum factors which bind cellular receptors which interact with Jak kinase (JAK), and the signal transducer and activator of transcription (STAT) proteins for signal transduction can replace the Ba/F3 cell line to otherwise provide a similar assay system for monitoring avian leptin levels, agonists and antagonists thereof. This assay can be used to screen avian live-stocks for individuals exhibiting exceptionally high or exceptionally low blood leptin levels. Some of these individuals are expected to include leptin or leptin-receptor gene mutations leading to either up or down regulation. Such mutant avians can be employed for selected breeding and the mutated genes thereof can be isolated.

The polynucleotide and recombinant protein according to the present invention can provide a tool for cloning avian leptin genes and/or isolating avian leptin proteins by means of, for example, affinity chromatography, yeast two hybrid system, or screening of expression libraries with labeled receptor protein. It will be appreciated in this respect that the effect of leptin or leptin agonists on the onset of puberty, could significantly increase the profitability in the egg industry and breeder flocks.

It is expected that intervention with the endogenous activity of avian leptin using an avian leptin-receptor will provide a replacement for the forced molting procedure which is employed to improve performance and profitability of old laying hens (Rolon *et al.*, 1993; Hurwitz *et al.*, 1995). The forced molting procedure is typically effected by a short period of feed withdrawal, which results in cessation of production, involution of the reproductive tract, and loss of primary feathers (as reviewed by Brake, 1993).

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be

limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturers' specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, 1989, which is incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

In an attempt to elucidate whether avians posses a mechanism for regulating appetite and other aspects of energy balance, such as, but not limited to, food consumption, energy expenditure, puberty, fertility and immunity, which is similar to that found in mammals, and while reducing the present invention to practice, the cloning of a chicken leptin-receptor has been exercised.

Preparing human leptin-receptor probe for screening libraries: To clone a chicken leptin-receptor homolog gene, a probe was generated according to a sequence corresponding to conserved regions of mammalian leptin-receptor genes, which are expected to encode active domains, and as

such are likely to be more preserved across the animal kingdom, including in avians.

According to these guidelines, a fragment of about 1 kb which includes exons 6 to 13 of the human leptin-receptor gene was generated by reverse transcriptase - polymerase chain reaction (RT-PCR) from human abdominal fat mRNA using human exon 6 forward (13F) 5'-GTCAGTTCAGCCCATAAATATGG-3' (SEQ ID NO:1) and human exon 13 reverse (13R) 5'-TTGCATCATAAACCTCATACAT-3' (SEQ ID NO:2) primers. Following purification, the resulting RT-PCR fragment of the expected size, 1095 bp, was radioactively labeled with ^{32}P-CTP using the random priming kit from Boehringer Mannheim. Specific activity was approximately 10^8 cpm/μg DNA.

Screening a chicken adipose tissue cDNA library with a human leptin-receptor derived probe; extensive screening did not yield positive clones: A commercially available and highly representative lambda ZAP cDNA library prepared from adipose tissue of a seven week old broiler male (Staratagene, Cat. No. 935405) was plated on 80 mm plates, 8 X 10^5 plaques were blotted onto nitrocellulose filters using standard protocols (Sambrook *et al*, 1989) and subsequently screened using the human derived leptin-receptor probe described hereinabove. Several plaques, which appeared to be positive, were identified and isolated, however, secondary screening of these isolated plaques indicated that the signals obtained were false positive signals. Assuming that low level of sequence similarity might be the problem, the screen was repeated using improved and supposedly more suitable hybridization conditions. The membranes were pre-hybridized for two hours at 42 °C in 5 x SSC; 50 % formamide; 5 x Denhardt's solution; 10 % dextran sulfate; 0.1 % SDS; and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out overnight at 42 °C in the same solution but with addition of the above described ^{32}P-

labeled human derived probe. Hybridization under these improved conditions, also resulted only in false positive signals.

Screening a chicken genomic DNA library led to the isolation of a chicken leptin genomic fragment: A commercial chicken lambda FIXII genomic library from male white Leghorn chicken (Stratagene, Cat. No. 946401) was then screened with the human leptin-receptor probe described above. 500,000 plaques, roughly representing 5 genome equivalence of the chicken genome (estimating the chicken genome to be roughly 1 x 10^9 bp) were screened using the improved hybridization conditions detailed hereinabove. Three independent clones were identified and isolated.

As shown in Figures 1a, DNA extracted from one of the clones thus isolated (which is referred to herein as phage 1) was digested with several restriction endonucleases (Eco RI, Pvu II and Hind III) and combinations thereof. The restriction fragments were then size separated using gel-electrophoresis. As shown in Figure 1b, blotting the size separated restriction fragments onto a nitrocellulose filter, and subsequent hybridization thereof with the human leptin-receptor probe described hereinabove, yielded several specific bands in each of the lanes, which hybridized with the human derived probe.

A Pvu II-Eco RI fragment of 1340 bp (Figures 1a-b, SEQ ID NO:3) was then subcloned into a Blue-Script plasmid (Stratagene) and sequenced from the T3 and the T7 ends of the plasmid's cloning polylinker. Computerized comparison of the sequence with GeneBank sequences revealed sequence similarity to mammalian leptin-receptor exons 9 and 10 (SEQ ID NOs:4-5, respectively).

As shown in Figure 2, alignment of the newly isolated sequence with exons 9 and 10 of the human leptin-receptor gene sequence using the Smith-Waterman algorithm (and the following parameters: Matrix = Blosum62, gapopen = 10.0, and gapextend = 0.5) showed a 76 % nucleotide identity to the 291 bp human leptin-receptor exon 9 (SEQ ID NO:4) and a 75 %

nucleotide identity to the 118 bp human leptin-receptor exon 10 (SEQ ID NO:5). An intron of 160 bp (Figure 2, SEQ ID NO:3) separating the newly identified chicken leptin-receptor exons 9 and 10 showed no sequence similarity to any GeneBank sequence.

Cloning and sequencing the full length cDNA:

Re-screening the chicken cDNA library using a chicken derived probe; extensive screening did not yield positive clones: Since the leptin-receptor gene in mammals is estimated to be about 100 kb long, the option of "chromosome walking" was ruled out at first. Assuming that using a chicken specific probe would increase sensitivity, a preferred approach of re-screening the chicken adipose cDNA library, this time with a chicken leptin-receptor derived probe was adopted.

To this end, mRNA derived from chicken adipose tissue (Leghorn, female) was reverse transcribed using the superscript cnzyme (Stratagene) and a random primer (Gibco-BRL), and subsequently PCR amplified using a forward primer derived from chicken exon 9 (9F) - 5'-AAGATACTGACCAGTGTTGGTTC-3' (SEQ ID NO:6); and reverse primer derived from chicken exon 10 (10R) - 5'-ATGCGTTTGGGTTTGCAGACCAT-3' (SEQ ID NO:7). In addition, a pair of nested primers was used for nested PCR amplification - 5'-TGTTTGGTGGCTGAATTTAGCAG-3' (SEQ ID NO:8) and 5'-GTTAAGTACCCATCAGTTTCACAT-3' (SEQ ID NO:9).

Only following amplification with the nested primers, a 230 bp band detectable by ethidium bromide staining was obtained, isolated and verified by DNA sequencing to include a chicken leptin-receptor derived sequence.

Due to the small size of the DNA fragment thus isolated and in order to avoid generation of fragments shorter than 230 bp, labeling was performed using the nested PCR primers and the Klenow fragment (New England BioLabs). The specific activity of the resulting probe was 10^8 cpm/g DNA.

This probe was used to re- screen the lambda ZAP cDNA library (Staratagene, Cat. No. 935405) as described hereinabove, again yielding only false-positive results.

Generating a longer cDNA probe: In order to achieve a longer cDNA probe of the chicken leptin-receptor gene, a 7 kb Eco RI fragment of the genomic clone described above (see Figure 1) was isolated and subcloned into a Blue-Script vector. This 7 kb fragment was subsequently sequenced from the T7 and T3 ends of the plasmid's cloning polylinker. Computerized comparison of the sequence with GeneBank sequences revealed 63 % sequence identity to human leptin-receptor exon 15.

mRNA derived from chicken adipose tissue (Leghorn, female) was reverse transcribed using the superscript enzyme (Stratagene) and a random primer (Gibco-BRL), and subsequently PCR amplified using the forward primer 9F from chicken exon 9 and a reverse primer derived from chicken exon 15 (15R) - 5'-CACGGCTAGAATTATAATGGTGT-3' (SEQ ID NO:10).

In a subsequent amplification an additional forward nested primer (SEQ ID NO:8) was used in combination with the above reverse primer 15R to yield a 1 kb amplification product which was subsequently isolated and sequenced, and was shown by sequence comparison analysis to include sequences corresponding to exons 9-15 of leptin-receptor genes.

Re-screening the chicken cDNA library using the longer chicken derived probe (exons 9-15); extensive screening did not yield positive clones: The above described 1 kb fragment was randomprime labeled as described hereinabove and served as a probe to re-screen the lambda ZAP cDNA library (Staratagene, Cat. No. 935405) as described hereinabove, again yielding only false-positive results.

Amplification of cDNA from the lambda ZAP cDNA library; extensive effort did not yield positive amplification products: At this point another approach to isolate a longer chicken leptin-receptor cDNA was

attempted. Employing nested chicken leptin-receptor specific primers and vector specific primers, DNA extracted from the adipose tissue cDNA library (Staratagene, Cat. No. 935405) was PCR amplified in an effort to isolate chicken leptin-receptor sequences. To this end, 0.5 μg of purified library phage DNA, which roughly represents the DNA content of 10^{10} phage particles, which represent several folds of the RNA complexity was employed. DNA was extracted from 100 μl of the original library, and 0.5 μg DNA was used in a PCR reaction with specific chicken leptin-receptor primers (exon 10 reverse (10R) or exon 15 forward (15F)) in combination with primers from the cloning linker of the lambda ZAP-phage (Blue Script cloning polylinker primers BST3 - 5'-CGCCAAGCTCGAAATTAA CCCTCACTA-3' (SEQ ID NO:11) and BST7 - 5'-GCCAGTGAATTGTAATACGACTCACTA-3' (SEQ ID NO:12).

As shown in the negative image of Figure 6, in some cases, bands stainable by ethidium bromide, smaller than 2 kb were detected, yet their purification and subsequent sequencing resulted in unknown sequences.

Rapid amplification of cDNA ends (RACE); extensive effort did not yield positive amplification products: As reported above, screening the lambda ZAP cDNA library (Staratagene, Cat. No. 935405) with several different probes failed to result in positive isolates, suggesting that the chicken leptin-receptor cDNA is either not represented or represented in very low abundance in the library, possibly due to low abundance of leptin-receptor mRNA in chicken adipose tissue.

Cloning a full length chicken leptin-receptor cDNA was then attempted via the rapid amplification of cDNA ends (RACE) approach, using the highly recommended Marathon kit (Clontech).

Chicken abdominal fat and hypothalamic tissue mRNA samples, both tissues are known to highly express leptin-receptor in mammals, were prepared.

To this end, hypothalamic RNA was extracted from a pool of 30 hypothalamuses of females White-Leghorn chickens. Abdominal fat RNA was extracted from pooled fat derived from two of these females. Both fat and hypothalamus RNA preparations were examined for the presence of leptin-receptor mRNA. As shown in Figure 3, employing chicken leptin-receptor exon 9 forward primer (9F) and exon 15 reverse primer (15R), both RNA preparations directed the formation of low amounts of RT-PCR products, which RT-PCR products were detectable via hybridization with a chicken leptin-receptor probe corresponding to exons 9-10. Furthermore, as shown in Figures 4a-b, the RNA preparation from hypothalamus was subjected to Northern blot analysis using the chicken leptin-receptor probe corresponding to exons 9-15 and yielded two bands of about 5 and 7 kb corresponding in size to the short and long alternatively splices transcripts of the mammalian leptin-receptor genes.

PolyA$^+$ RNA molecules were enriched from both these preparations using a mRNA isolation kit (Boehringer Mannheim, Cat. No. 1741985).

For 5' RACE an exon 10 reverse primer (10R) 5'-ATGCGTTTGGGTTTGCAGACCAT-3' (SEQ ID NO:13) and an exon 10 reverse nested primer (10NR) 5'-GTTAAGTACCCATCAGTTTCACAT-3' (SEQ ID NO:14) were employed. For 3' RACE exon 15 forward primer (15F) 5'-GCACCACCTGCTCATTTCCATGGA-3' (SEQ ID NO:15) and exon 15 forward nested primer (15NF) 5'-CACACCATTACAATTCTAGCCGTGA-3' (SEQ ID NO:16) were employed. 5' and 3' RACE reactions were performed following the manufacturer's instructions.

In both 5' and 3' RACE, first amplifications resulted in ethidium bromide stainable smears, whereas second (nested) amplifications yielded faint ethidium bromide stainable bands. Figure 6 shows a representative example.

Subsequent amplification, purification and sequencing of the bands appeared in any of the RACE experiments resulted in either unidentified or non-relevant sequences.

Modifications to the original RACE technique, still did not lead to amplification of chicken leptin-receptor sequences: It is estimated that the difficulty of cloning the chicken leptin-receptor cDNA by RACE was due to its low abundance. Therefore, additional nested primers were designed. For 5' RACE the following primers were employed: exon 15 reverse primer (15R), exon 10 reverse primer (10R) and then exon 10 reverse nested primer (10NR), For 3' RACE the following primers were employed: exon 10 forward primer (10F) - 5'-ATGGTCTGCAAACCCAAACGCAT-3' (SEQ ID NO:17), exon 15 forward primer (15F) and the exon 15 forward nested primer (15NF). This strategy as well resulted only in false bands.

Fishing for chicken leptin-receptor mRNA molecules from a hypothalamic RNA preparation failed to provide chicken leptin-receptor mRNA: Fishing of chicken leptin-receptor mRNA molecules from a hypothalamic RNA preparation was attempted using a chicken leptin-receptor specific oligonucleotide linked to biotin. To this end, hypothalamuses of 30 female Leghorn chickens were pooled, then checked for the expression of chicken leptin-receptor by Northern blot analysis and incubated with a biotinylated chicken leptin-receptor oligonucleotide derived from exon 9 - 5'- Biotin-CTAAATTCAGCCACCAAA-3' (SEQ ID NO:18). Fishing was then performed using streptavidin coated magnetic particles and a magnetic separator (Boehringer Mannheim). A RACE library prepared from this chicken leptin-receptor enriched mRNA failed to lead, however, to amplification of chicken leptin-receptor cDNA sequences.

Screening the genomic library with probes derived from the 5' and the 3' regions of the mouse leptin-receptor gene; extensive screening did not yield positive clones: As so far, the only isolatable chicken leptin-receptor sequences were derived from the chicken genomic library

described herein. Assuming at this point that the chicken leptin-receptor transcripts are similar to mammalian leptin-receptor transcripts in global exon-intron structure, it was decided to try and isolate genomic clones including exons 3 and 20, which are the first and last translated exons in the longest transcript of the human leptin-receptor gene, and to use the sequence information therefrom to set up an RT-PCR reaction which will provide a cDNA which corresponds to the entire translated sequence of the chicken leptin-receptor gene. To this end, mouse specific leptin-receptor primers: mouse exon 3 forward 5'-TGTGGTTTTGTTACACTGGG-3' (SEQ ID NO:19); mouse exon 4 reverse 5'-TCTTCCCTTCAGTGTTGTCT-3' (SEQ ID NO:20); mouse exon 20 forward 5'- CAAGCATGCAGAATCAGTGA-3' (SEQ ID NO:21); and mouse exon 20 reverse 5'-CAAAGTGTGAGCATCTCTCC-3' (SEQ ID NO:22), were prepared according to the published sequence and were used in an RT-PCR reaction to amplify cDNAs corresponding to exons 3, 4 and 20 of the mouse leptin-receptor gene. Thus, mouse (CD1 strain) abdominal fat total RNA was extracted and was thereafter subjected to RT-PCR. Reverse transcription was effected by the superscript enzyme (Stratagene) and a random primer (Gibco-BRL), followed by 35 PCR cycles: 94 °C, 30 seconds, 55 °C, 30 seconds, 72 °C 1 minute, to yield cDNA bands of the expected sizes 310 bp for exons 3 and 4 and 671 bp for exon 20. It will be recalled in this respect that in order to RT-PCR chicken leptin-receptor derived sequences, nested PCR reactions were necessary. The identity of the mouse exons 3 and 20 cDNA probes thus generated was confirmed by DNA sequencing. An initial hybridization was employed to examine whether the genomic DNA clones so far isolated contain the cDNA ends, however, no positive signals were obtained even under the lowest stringency wash conditions. Hybridization was effected in 5 x SSC; 50 % formamide, 5 x Denhardt's solution, 10 % dextran sulfate, 0.5 % SDS and 100 g/ml denatured salmon sperm DNA at 42 °C. Final wash was with 1 x

SSC and 0.1 % SDS at 42 °C. Therefore, the genomic library was re-screened as described above with each of the exon 3 and exon 20 mouse derived probes, however, no positive clones were obtained.

Amplifying chicken leptin-receptor sequences of genomic clones with chicken leptin-receptor primers and vector derived primers: Two PCR amplifications with chicken leptin-receptor specific primers (exon 9 reverse (9R) and exon 15 forward (15F) primers) combined with primers compatible with the phage cloning polylinker, phT7 - 5'-AATACGACTCACTATAGGGCGTCG-3' (SEQ ID NO:23); and phT3 - 5'-CGCGAGCTCAATTAACCCTCACTA-3' (SEQ ID NO:24), respectively, with enzymes recommended for long template PCR amplifications (e.g., Expand long template PCR system and Expand high fidelity PCR system both from Boehringer Mannheim) were independently attempted on DNA extracted from the above described genomic clones (phage 1 and phage 2). However, bands of different sizes were obtained in both reactions and were analyzed to be false-positives.

Surprisingly, and as shown in Figure 7, while using the Super-term DNA polymerase from SR-PRODUCTS, UK, Cat. No. SRP-801 which is not at all recommended for long template PCR as a PCR enzyme, a similar reaction setup resulted in orientation specific chicken leptin-receptor bands. Cycling conditions were 2 minutes at 94 °C, followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 5 seconds and 68 °C for 12 minutes. The dNTP's concentration employed was 0.5 mM. The ethidium-bromide detectable bands were purified using the Wizard PCR purification kit (Promega). Sequencing the 14 kb band derived from phage 1 with the phT7 primer (see Figure 8, phage 1) led to identification of exon 20 of the chicken leptin-receptor gene. This sequence was used to prepare an exon 20 reverse primer (20R) - 5'-AAGCCCACCTGATCTGGAGTTA-3' (SEQ ID NO:25) and in combination with the exon 15 forward primer (15F) described above

was employed in an RT-PCR to obtain a cDNA covering exons 15-20. This cDNA was subsequently sequenced.

Using the same experimental procedure, exon 6 was identified on another phage previously isolate (Figure 8, phage 2) and led to the completion of the sequence from exon 6 to exon 20 using RT-PCR as described, employing this time the above exon 20 reverse primer (20R) in combination with an exon 6 forward primer (6F) - 5'-ACTGGGAGATACATCTACCAGCA-3' (SEQ ID NO:26).

Completing cloning and sequencing of the full length chicken leptin-receptor cDNA: Thus, the isolation of exons 6-20 of the chicken leptin-receptor cDNA was effected by identifying sequences derived from extreme exons present on genomic clones, followed by RT-PCR bridging. The chicken leptin-receptor gene structure shares low homology with mammalian corresponding genes, nevertheless, the Exon-intron structure thereof is preserved. It is therefore assumed that 3 additional exons (3-5) are necessary for completing the entire coding sequence, which exons are expected to include the start signal for translation and to have a total length of about 500 bp.

To this end, a probe containing sequences derived from chicken leptin-receptor exon 6 and intron 5 was prepared using DNA of phage 2 (Figure 8) as a template in a PCR reaction effected via the phT7 and an exon 6 reverse primers (6R) - 5'-ACATCAAGGGTGACCAAA-3' (SEQ ID NO:29). This probe was used to re-screen the genomic library. The screening resulted in the identification and isolation of two positive clones (Figure 10, clones 3 and 4).

These clones were further characterized as described hereinbelow. Phage 4 hybridized with a probe derived from exon 6, yet failed to hybridize with a probe derived from exons 9 to 10. Clone 3, on the other hand, hybridized with both of these probes. Direct sequencing of clone 4 purified

DNA with the phT3 primer indicated that clone 4 includes sequences of exon 6 and sequences present upstream of exon 6.

Phage 4 failed to hybridize with a probe corresponding to mouse exons 3 and 4. This is due to a low sequence homology between the mouse and chicken sequences as was confirmed by partial sequence analysis of phage 4 DNA which indicated that this phage includes at least exons 4 and 5, and most likely, based on the overall size thereof, also exon 3 of the chicken leptin receptor gene.

Sequencing fragments derived from phage 4 (Figure 8) led to the identification of exons 5 and 4. RACE procedure with exon 6 reverse primer (6FII) 5'-CACATGGCACTCACATTTGCCATA-C-3' (SEQ ID NO:30) and a nested exon 4 forward primer (4R): 5'-CGGCAGCAGAAGGACTCGAAGATG-3' (SEQ ID NO:31) led to identification of exon 3 and the ATG translation initiation codon which was found at the expected position as is compared to corresponding mammalian cDNAs.

Figure 9a shows the nucleotide sequence of exons 3-20 of the chicken leptin-receptor gene (SEQ ID NO:27). The translation of this sequence into amino acids is shown in Figure 9b (SEQ ID NO:28).

Tables 1 and 2 below show sequence homologies shared among exons 6-20 of the leptin-receptor genes of mouse (*Mus musculus*), rat (*Rattus norvegicus*), human (*Homo sapiens*), porcine (*Sus scrofa*), and chicken (*Gallus domesticus*). Please note the low sequence homology shared between the chicken leptin-receptor and the corresponding mammalian genes (59-62 % identical nucleotides; 49-51 % identical amino acids; 75-78 % identical or conserved amino acids), as compared to the much higher homology shared among the mammalian genes (80-92 % identical nucleotides; 74-91 % identical amino acids; 90-96 % identical or conserved (similar) amino acids)

TABLE 1

Comparison of leptin-receptor amino acid sequences, from human, porcine, rat, mouse and chicken

	HS	*SS*	*RN*	*MM*	*GD*
HS	100%	84% (94%)	75.56% (90%)	75% (90%)	50% (76%)
SS		100%	74% (90%)	75% (90%)	51% (78%)
RN			100%	91% (96%)	50% (75%)
MM				100%	49% (75%)
GD					100%

HS - Homo sapiens; SS - Sus scrofa; RN - Rattus norvegicus; MM - Mus musculus; and GD - Gallus domesticus (exons 6-20).

* In parenthesis % similarity, representing conservative changes in amino acids, as determined, for example, according to the following matrix:

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	i	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+
R	-	i	+	-	-	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-
N	-	+	i	+	-	+	+	+	+	-	-	+	-	-	-	+	+	-	-	-
D	-	-	+	i	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-
C	+	-	-	-	i	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Q	-	+	+	+	-	i	+	-	+	-	-	+	+	-	-	+	-	-	-	-
E	-	+	+	+	-	+	i	-	+	-	-	+	-	-	-	+	-	-	-	-
G	+	-	+	-	-	-	-	i	-	-	-	-	-	-	-	+	-	-	-	-
H	-	+	+	-	-	+	+	-	i	-	-	-	-	-	-	-	-	-	+	-
I	-	-	-	-	-	-	-	-	-	i	+	-	+	+	-	-	-	-	-	+
L	-	-	-	-	-	-	-	-	-	+	i	-	+	+	-	-	-	-	-	+
K	-	+	+	-	-	+	+	-	-	-	-	i	-	-	-	+	-	-	-	- +
M	-	-	-	-	-	+	-	-	-	+	+	-	i	+	-	-	-	-	-	+
F	-	-	-	-	-	-	-	-	-	+	+	-	+	i	-	-	-	+	+	-
P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	i	-	-	-	-	-
S	+	-	+	+	-	+	+	+	-	-	-	+	-	-	-	i	+	-	-	-
T	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	i	-	-	+
W	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	i	+	-
Y	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	i	-
V	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	-	-	i

i = identical aa

- = non similar aa

+ = similar aa

TABLE 2

Comparison of leptin-receptor nucleotide sequences, from human, porcine, rat, mouse and chicken

	HS	*SS*	*RN*	*MM*	*GD*
HS	100%	87%	81%	81%	62%
SS		100%	80%	78%	61%
RN			100%	92%	59%
MM				100%	59%
GD					100%

HS - Homo sapiens; SS - Sus scrofa; RN - Rattus norvegicus; MM - Mus musculus; and GD - Gallus domesticus (exons 6-20).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

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WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a nucleic acid sequence at least 70 % identical with a nucleic acid encoding a polypeptide as set forth in SEQ ID NO:28 or a portion thereof longer than 30 contiguous amino acids, as determined using the Smith-Waterman algorithm and the following parameters: Matrix = Blosum62, gapopen = 10.0, and gapextend = 0.5.

2. The isolated polynucleotide of claim 1, wherein said nucleic acid sequence is as set forth in SEQ ID NO:27 or a portion thereof longer than 90 contiguous nucleotides and natural and man induced variants thereof.

3. The isolated polynucleotide of claim 1, wherein said nucleic acid sequence is of an avian.

4. The isolated polynucleotide of claim 1, wherein said polypeptide is capable of binding leptin under physiological conditions.

5. The isolated polynucleotide of claim 1, wherein said nucleic acid sequence is as set forth in SEQ ID NO:27.

6. The isolated polynucleotide of claim 1, further comprising a vector ligated to said nucleic acid sequence.

7. The isolated polynucleotide of claim 1, wherein said nucleic acid sequence is a product of a polymerase chain reaction.

8. A genetically modified cell expressing the polynucleotide of claim 1 in a sense or antisense orientation.

9. An isolated polynucleotide comprising a nucleic acid sequence hybridizable with a nucleic acid encoding a polypeptide as set forth in SEQ ID NO:28 under a hybridization selected from the group consisting of stringent hybridization and moderate hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC, 5 x Denhardt's solution, 1 % SDS and 100 g/ml denatured salmon sperm DNA, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, whereas

moderate hybridization is effected by a hybridization solution of 6 x SSC, 5 x Denhardt's solution, 1 % SDS and 100 g/ml denatured salmon sperm DNA, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C.

10. The isolated polynucleotide of claim 9, wherein said nucleic acid sequence is as set forth in SEQ ID NO:27 or a portion thereof longer than 90 contiguous nucleotides and natural and man induced variants thereof.

11. The isolated polynucleotide of claim 9, wherein said nucleic acid sequence is of an avian.

12. The isolated polynucleotide of claim 9, wherein said polypeptide is capable of binding leptin under physiological conditions.

13. The isolated polynucleotide of claim 9, wherein said nucleic acid sequence is as set forth in SEQ ID NO:27.

14. The isolated polynucleotide of claim 9, further comprising a vector ligated to said nucleic acid sequence.

15. The isolated polynucleotide of claim 9, wherein said nucleic acid sequence is a product of a polymerase chain reaction.

16. A genetically modified cell expressing the polynucleotide of claim 11 in a sense or antisense orientation.

17. A recombinant protein comprising an amino acid sequence at least 70 % homologous with SEQ ID NO:28 or a portion thereof longer than 30 contiguous amino acids, as determined using the Smith-Waterman algorithm and the following parameters: Matrix = Blosum62, gapopen = 10.0, and gapextend = 0.5.

18. The recombinant protein of claim 17, wherein said amino acid sequence is as set forth in SEQ ID NO:28 and natural and man induced variants thereof.

19. The recombinant protein of claim 17, wherein said amino acid sequence is of an avian.

20. The recombinant protein of claim 17, wherein said polypeptide is capable of binding leptin under physiological conditions.

21. The recombinant protein of claim 17, wherein said amino acid sequence is as set forth in SEQ ID NO:28.

22. A single stranded polynucleotide of at least 18 nucleotides hybridizing with a nucleic acid sequence as set forth in SEQ ID NO:27 under a hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 g/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 1 - 1.5 °C below the T_m, final wash solution of 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS at 1 - 1.5 °C below the T_m; moderate hybridization is effected by a hybridization solution of 6 x SSC and 0.1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 g/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 2 - 2.5 °C below the T_m, final wash solution of 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS at 1 - 1.5 °C below the T_m, final wash solution of 6 x SSC, and final wash at 22 °C; whereas mild hybridization is effected by a hybridization solution of a hybridization solution of 6 x SSC and 1 % SDS or 3 M TMACI, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 g/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 37 °C, final wash solution of 6 x SSC and final wash at 22 °C.

23. A genetically modified cell expressing the recombinant protein of claim 17.

24. A single stranded polynucleotide of at least 18 contiguous nucleotides of SEQ ID NO:27.

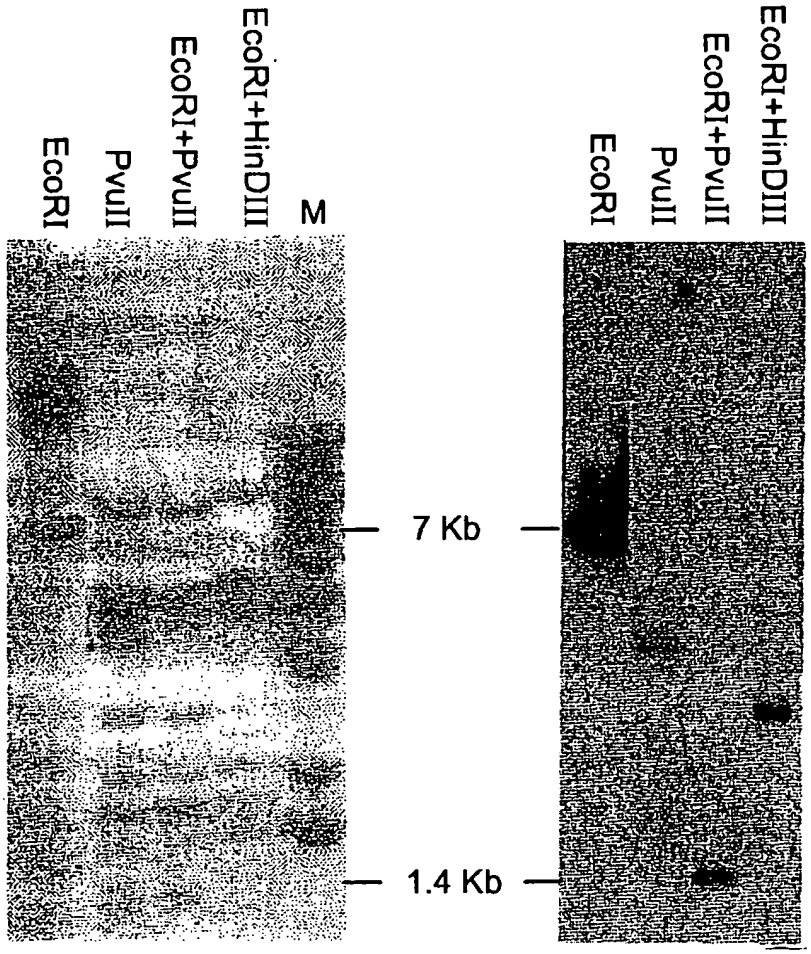
EcoRI
PvuII
EcoRI+PvuII
EcoRI+HinDIII
M
7 Kb
1.4 Kb

Fig. 1a

EcoRI
PvuII
EcoRI+PvuII
EcoRI+HinDIII

Fig. 1b

SEQ ID NO

```
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  71 ATTAGCATTA AATGATAAAT GCATGGAGAC TGCGCGGTGA TCTCCATGAC ACCCCCATGT CTGGCCCTAT    140
 141 TGCTTTCAGA CCCAGGAGAA GCTGGGCTTT TCACTTATGC TGCCGTGTGT TGTTTTCAAT GTACTGCCCG    210
 211 GGTGTTTTCT GTCAGTTCAT NACCATTTGC CTAGGGACAG TAAAGGTTAT TTTTATTAGA ACTTGTAAAC    280
 281 ATGAGTTATG GAATGAAACA GTCAAGGATC TGTTCCTGCT CCTCTGCCCT ATAGCAATTC GTAATAACAA    350
 351 ACCTGGAAGA ACAGATGATG TAGAGATACT ACGGGATAAT AGGAATTCTC TGTNGCTCTG TAGTTTTTGT    420
 421 TGTTGTTCTG ATTCTGATCT CAGTGGATTT CTTTCTTTTC TTGTAGCTGA AAGTGCTGTA CTTCCCTACT    490
   1                                          HUMAN EXON 9 ATGTCATATA CTTTCCACCT     20
 491 AAGATACTGA CCAGTGTTGG TTCTAACGTT TCGTTTCATT GCATCTATAA AAACAAAACC CAGAGCGTAG    560
  21 AAAATTCTGA CAAGTGTTGG GTCTAATGTT TCTTTTCACT GCATCTATAA GAAGGAAAAC AAGATTGTTC     90
 561 CTTCCAAGAA AGATTGTTTG GTGGCTGAAT TTAGCAGAAG AAATCCCAGA AAGTCAGTAT ACGCTTGTGA    630
  91 CCTCAAA_AG AGATTGTTTG GTGGATGAAT TTAGCTGAGA AAATTCCTCA AAGCCAGTAT GATGTTGTGA    159
 631 ACGATCGCGT AAGCAAAGTT ACTCTTTTCA ACTTGAAAGC AACCAAACCT AGAGGAAGTT TCTTCTATAA    700
 160 GTGATCATGT TAGCAAAGTT ACTTTTTTCA ATCTGAATGA AACCAAACCT CGAGGAAAGT TTACCTATGA    229
 701 CGCATTGTAC TGTTGCCATC AAAATAGGGA ATGCCATCAT AGATACGCTG AATTATATGT AGTAGGTAAG    770
 230 TGCAGTGTAC TGCTGCAATG AACAT___GA ATGCCATCAT CGCTATGCTG AATTATATGT GATTG         291
 771 AGTCCAAATG ATTTTAAATT ACAGTTTCGG TGCATTTTGA TAGTAAGGTC GATCTTCTTA AACTCCACGT    840
 841 AGCTTTTATC AAATAATCAC AGTTTGTAAT GCATGCTTTT TGTGTAAACA AATATATCTT ATTAATAACT    910
 911 TCTAATGTGT TTTTAATTAG ATGTGAATAT CAATATCAAA TGTGAAACTG ATGGGTACTT AACTAAAATG    980
   1       HUMAN EXON 10  ATGTCAATAT CAATATCTCA TGTGAAACTG ATGGGTACTT AACTAAAATG     50
 981 ACTTGCAGAT GGTCTGCAAA CCCAAACGCA TTGCTCTTGG GGAGTTCCTT GCAGTTAAAA TACCACAGGT   1050
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1121 AGGAATAGAT GATAAAATGT GTGAATACAG AATGTGCTCA CTTCCTGTAA GCCAGACTTT TCCTCTGTTG   1190
1191 GGAACTCCAG AGGTATTGAA CATGTGTGCA AATGAGGGCT GTGGGTATCT GAGTAAGTGA TGTGGATTTT   1260
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1331 AGTCTAAAAC                                                                    1340
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Fig. 2

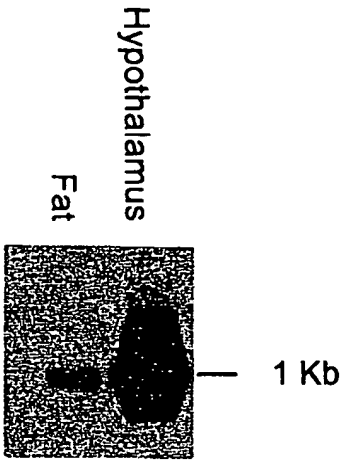

Fig. 3

Fig. 4a

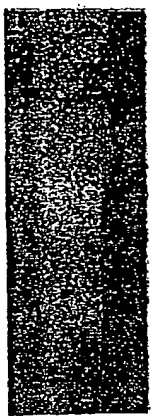

Fig. 4b

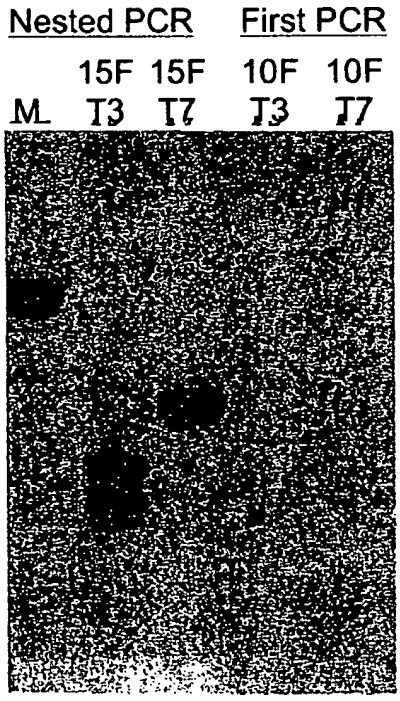

Fig. 5

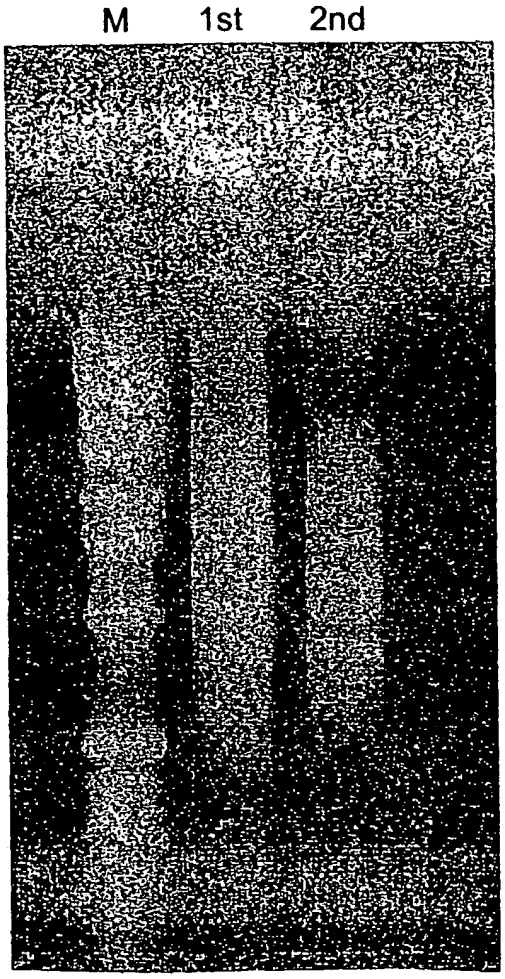

Fig. 6

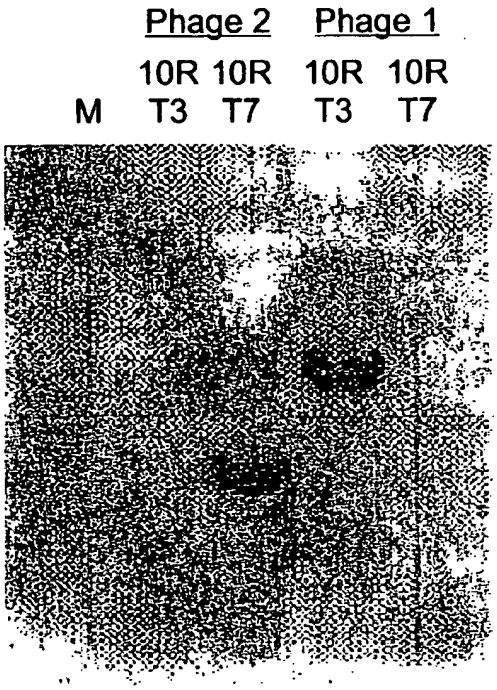

Fig. 7

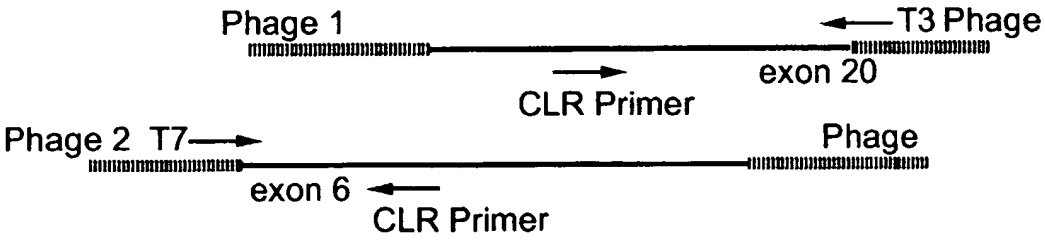

Fig. 8

SEQ ID NO:27

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CATGTCTTGCTGTTGG

exon 4
GGGCTTTCTTCACGTGGCAGCTGCCCACTGTATGGTCCACGAGATTCATCCCAGGAGCTT
TACATTACCGTGCCTACTGCTGAATGAGACATCTTCGAGTCCTTCTGCTGCCGGAGTTGT
ACGGAGCCGGTCTGGCTGGAGGAGGGAGCATGGAGTTGCTGAAACCAACCCTTCCTTGCT
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GTACAGTGCAAACATGCAAGCGAGGATGCTTATTCCTTCAGAAATGAGCATCTCTGCATC
TCAGGAAAGAG

exon 5
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exon 6
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GATGTCAGTCAAGCCCATAGACATAG

exon 7
TAAAGCCTGACCTCCCTTTGAATGTGCGTCTAGAGATGACAGAGAGAGGTCAAGTCAAGA
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GGAAATTCAGATCAAATGACTGGCAGGGA

exon 8
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exon 9
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exon 10
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exon 11
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exon 12
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Fig. 9a

exon 13
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exon 14
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AAGAACGTTACGCTCCTGTGGAAG

exon 15
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ACTGAAAGCACACACACCATTACATTTCTAGCCGTGAATTCAATTGGAGCTTCTTCAGTT
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exon 16
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AACAAAGAAGAGGAGATGAAGTGGGTGCAAGTTCCTCCAAATATTAGTAAACACTATATT
TAT

exon 17
GACCACTTTATTCTGATTGAGAAGTACCGGTTCAGCCTGTACCCCGTGTTTGCTGCAGGA
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exon 18
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exon 19
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exon 20
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TTTATATAATTTATATAAAAATATATTCAAAGGATGTCTGTTGGCCATGCAGACCCAAGC
ACGCGTCTCCCCATTCAGGAGTAAAGCATCTCTTTATACGTGCTGCCAGACTCTGAGTGA
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TAGGAGTCG

Fig. 9a (continued)

```
 37 ATGTATCATCAAATCATTCTGACCATGTCTTTGCTGTTGGGCTTTCTTCA 86 SEQ ID NO:27
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  1 MetTyrHisGlnIleIleLeuThrMetSerLeuLeuLeuGlyPheLeuHi 17 SEQ ID NO:28

 87 CGTGGCAGCTGCCCACTGTATGGTCCACGAGATTCATCCCAGGAGCTTTA 136
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 17 sValAlaAlaAlaHisCysMetValHisGluIleHisProArgSerPheT 34

137 CATTACCGTGCCTACTGCTGAATGAGACATCTTCGAGTCCTTCTGCTGCC 186
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 34 hrLeuProCysLeuLeuLeuAsnGluThrSerSerSerProSerAlaAla 50

187 GGAGTTGTACGGAGCCGGTCTGGCTGGAGGAGGGAGCATGGAGTTGCTGA 236
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 GlyValValArgSerArgSerGlyTrpArgArgGluHisGlyValAlaGl 67

237 AACCAACCCTTCCTTGCTAATGGATGAGGAGAGTTTTCTGTGTTGCCTTT 286
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 67 uThrAsnProSerLeuLeuMetAspGluGluSerPheLeuCysCysLeuT 84

287 GGAGCAATAACAACGCCAGTTGTTCTTTGTACAGTGCAAACATGCAAGCG 336
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 84 rpSerAsnAsnAsnAlaSerCysSerLeuTyrSerAlaAsnMetGlnAla 100

337 AGGATGCTTATTCCTTCAGAAATGAGCATCTCTGCATCTCAGGAAAGAGA 386
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 ArgMetLeuIleProSerGluMetSerIleSerAlaSerGlnGluArgAs 117

387 TTCAAATTGGAACATTGAATGTTGGGTTGAAGGCAAGCTGGATTTGTTAG 436
    ||||||||||||||||||||||||||||||||||||||||||||||||||
117 pSerAsnTrpAsnIleGluCysTrpValGluGlyLysLeuAspLeuLeuV 134

437 TTTGTAGCCTGCAGTTCCCGAAGTTTCACATGAGACTTGATATGAAGGTT 486
    ||||||||||||||||||||||||||||||||||||||||||||||||||
134 alCysSerLeuGlnPheProLysPheHisMetArgLeuAspMetLysVal 150

487 CATCTTTTATATGCTGTGTCAGAGCTGTCACTGGGAGATACATCTACCAG 536
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 HisLeuLeuTyrAlaValSerGluLeuSerLeuGlyAspThrSerThrSe 167

537 CACCCTGAAGAGGACTGCCCTGGCTGCTCAGTGTAACTGCAGTGAGTATG 586
    ||||||||||||||||||||||||||||||||||||||||||||||||||
167 rThrLeuLysArgThrAlaLeuAlaAlaGlnCysAsnCysSerGluTyrG 184

587 GCAAATGTGAGTGCCATGTGCCTTCCCCAAGACTCAACCACACTTACGTC 636
    ||||||||||||||||||||||||||||||||||||||||||||||||||
184 lyLysCysGluCysHisValProSerProArgLeuAsnHisThrTyrVal 200

637 ATGTGGCTGAAGACTGGGATTGGTCTAACACCTGTTTGGTCACCCTTGAT 686
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 MetTrpLeuLysThrGlyIleGlyLeuThrProValTrpSerProLeuMe 217

687 GTCAGCCAAGCCCATAGACATAGTAAAGCCTGACCTCCCTTTGAATGTGC 736
    ||||||||||||||||||||||||||||||||||||||||||||||||||
217 tSerAlaLysProIleAspIleValLysProAspLeuProLeuAsnValA 234
```

Fig. 9b

```
737 GTCTAGAGATGACAGAGAGAGGTCAAGTCAAGATCTGCTGGTCTGAGCCT 786
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
234 rgLeuGluMetThrGluArgGlyGlnValLysIleCysTrpSerGluPro 250

787 GTACCGATGCCGTACCCGCTCCGGTGTGAAGTGAACATCTTCTGGAAATT 836
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
251 ValProMetProTyrProLeuArgCysGluValAsnIlePheTrpLysPh 267

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    ||||||||||||||||||||||||||||||||||||||||||||||||||||
267 eArgSerAsnAspTrpGlnGlyValValGlnValAlaLeuAsnThrSerL 284

887 TAGACATAGACAATATGCTGCTTGATTCTTCCTCCTTTGCTCAAGTGAGG 936
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
284 euAspIleAspAsnMetLeuLeuAspSerSerSerPheAlaGlnValArg 300

937 TGCAAGAGTATTTGTGTCCCCGGGTTCTGGAGTGAATGGAGCACACTGTA 986
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
301 CysLysSerIleCysValProGlyPheTrpSerGluTrpSerThrLeuTy 317

987 TAATCTGAATGGGGAGGTGCTGTACTTCCCTACTAAGATACTGACCAGTG 1036
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
317 rAsnLeuAsnGlyGluValLeuTyrPheProThrLysIleLeuThrSerV 334

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     ||||||||||||||||||||||||||||||||||||||||||||||||||||
334 alGlySerAsnValSerPheHisCysIleTyrLysAsnLysThrGlnSer 350

1087 GTAGCTTCCAAGAAGATTGTTTGGTGGCTGAATTTAGCAGAAGAAATCCC 1136
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
351 ValAlaSerLysLysIleValTrpTrpLeuAsnLeuAlaGluGluIlePr 367

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     ||||||||||||||||||||||||||||||||||||||||||||||||||||
367 oGluSerGlnTyrThrLeuValAsnAspArgValSerLysValThrLeuP 384

1187 TCAACTTGAAAGCAACCAAACCTAGAGGAAGTTTCTTCTATAACGCATTG 1236
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
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1237 TACTGTTGCCATCAAAATAGGGAATGCCATCATAGATACGCTGAATTATA 1286
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
401 TyrCysCysHisGlnAsnArgGluCysHisHisArgTyrAlaGluLeuTy 417

1287 TGTAGTAGATGTGAATATCAATATCAAATGTGAAACTGATGGGTACTTAA 1336
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
417 rValValAspValAsnIleAsnIleLysCysGluThrAspGlyTyrLeuT 434

1337 CTAAAATGACTTGCAGATGGTCTGCAAACCCAAACGCATTGCTCTTGGGG 1386
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
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1387 AGTTCCTTGCAGTTAAAATACCACAGCAAAATTTATTGTTCTAACTTTCC 1436
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
451 SerSerLeuGlnLeuLysTyrHisSerLysIleTyrCysSerAsnPhePr 467

1437 AAGTACTCCTCCAGAATCAGAGGTGAAAGAATGCCATTTCCAGAGGAATC 1486
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
467 oSerThrProProGluSerGluValLysGluCysHisPheGlnArgAsnH 484
```

Fig. 9b (continued)

```
1487 ATTCTTATGAGTGCACATTTCAGCCTGTTTTTCTTTTATCTGGACATACC 1536
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 484 isSerTyrGluCysThrPheGlnProValPheLeuLeuSerGlyHisThr 500

1537 ATGTGGATTGAGCTTAAGCACTCGCTGGGAACACTTGAATCCTCACCAAC 1586
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 501 MetTrpIleGluLeuLysHisSerLeuGlyThrLeuGluSerSerProTh 517

1587 TTGTGTCGTTCCAGCAGATGTGGTGAAGCCACTGCCTCCCTCCAACATTA 1636
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
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1637 AAGCAGAGATCACCAGAAACGATGGGCTGCTGAACGTGAGCTGGACAAAC 1686
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
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     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 567 nArgGluGluLeuThrTrpGluLeuTyrGluValLeuSerValProThrA 584

1787 GATCAGCTGTGATAGAAGTGCAACTTTGTGTTGAATATATTGTTCAGATC 1836
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 584 rgSerAlaValIleGluValGlnLeuCysValGluTyrIleValGlnIle 600

1837 CGCTGCAGAGCCCTGGATGGCTTAGGCTACTGGAGCAACTGGAGCAGATC 1886
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 601 ArgCysArgAlaLeuAspGlyLeuGlyTyrTrpSerAsnTrpSerArgSe 617

1887 AGCCTATGCAGCTGTAAAAGATATCCAAGCTCCCTTACATGGCCCTGAGT 1936
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 617 rAlaTyrAlaAlaValLysAspIleGlnAlaProLeuHisGlyProGluP 634

1937 TTTGGAGAACTGTCACTGAAGATCCAGCAACGGGGCAGAAGAACGTTACG 1986
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 634 heTrpArgThrValThrGluAspProAlaThrGlyGlnLysAsnValThr 650

1987 CTCCTGTGGAAGCCACTGATGAAGAATCACTCACTGTGCAGTGTGAGCCG 2036
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 651 LeuLeuTrpLysProLeuMetLysAsnHisSerLeuCysSerValSerAr 667

2037 GTACGTTATAAAGCATCAGACGTCAGAAAACACCTCGTGGTCAGARTATG 2086
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 667 gTyrValIleLysHisGlnThrSerGluAsnThrSerTrpSerGluTyrV 684

2087 TCGACAATGGCACCACCTGCTCATTTCCATGGACTGAAAGCACACACACC 2136
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 684 alAspAsnGlyThrThrCysSerPheProTrpThrGluSerThrHisThr 700

2137 ATTACATTTCTAGCCGTGAATTCAATTGGAGCTTCTTCAGTTAATTTTAA 2186
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 701 IleThrPheLeuAlaValAsnSerIleGlyAlaSerSerValAsnPheAs 717

2187 TTTAACTCTGTCACAACAAATGAGCACAGTGAATGCTGTGCAGTCTCTCA 2236
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 717 nLeuThrLeuSerGlnGlnMetSerThrValAsnAlaValGlnSerLeuI 734
```

Fig. 9b (continued)

```
2237 TTGCTTACCCAGTGAACAGCACGTGTGTGATTTTGACTTGGACGCTTTCG 2286
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 734 leAlaTyrProValAsnSerThrCysValIleLeuThrTrpThrLeuSer 750

2287 CCTCAAATATATGTGATAACATCTTTTATTATTGAGTGGAGAAACCTTAA 2336
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2387 ACTATATTTATGACCACTTTATTCTGATTGAGAAGTACCGGTTCAGCCTG 2436
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 784 isTyrIleTyrAspHisPheIleLeuIleGluLysTyrArgPheSerLeu 800

2437 TACCCCGTGTTTGCTGCAGGAGTTGGCAAATCCAGAGCCACGGATCAGTT 2486
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 801 TyrProValPheAlaAlaGlyValGlyLysSerArgAlaThrAspGlnPh 817

2487 CTCCAAAGATGGGTATGCCAGTCAGACCAGTTCTAACCTCTATATGGTCC 2536
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 817 eSerLysAspGlyTyrAlaSerGlnThrSerSerAsnLeuTyrMetValL 834

2537 TGCCAATAGTTATTTCAACCTCCGTGCTGTTGCTTGGAGCGCTGCTGGTT 2586
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 834 euProIleValIleSerThrSerValLeuLeuLeuGlyAlaLeuLeuVal 850

2587 TCGCACCGAAGAATGAAGAAACTGCTCTGGGAAGATGTTCCGATCCCCAA 2636
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 851 SerHisArgArgMetLysLysLeuLeuTrpGluAspValProIleProLy 867

2637 GAATTGCTCGTGGGCACAAGGTGTTGATTTTCAGCAGCCTGAAACTTTTG 2686
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 867 sAsnCysSerTrpAlaGlnGlyValAspPheGlnGlnProGluThrPheG 884

2687 AGCACCTTTTTTGTCAAGCACCCTGAAGCAATGTCATTTGAGCCTCTTCTT 2736
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 884 luHisLeuPheValLysHisProGluAlaMetSerPheGluProLeuLeu 900

2737 CTGGAACCGGAAATAGTGCTGGAAGACATCAGTGTTACTAAAGCTTTGGA 2786
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 901 LeuGluProGluIleValLeuGluAspIleSerValThrLysAlaLeuGl 917

2787 ACAAGAAGACACGCAAGATTTCTTAGCGCTTGATTCCATGTTCACAAAAC 2836
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
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2887 CGCAGCTCCATGGAGTGCTCCCCCAGTGACCCGACCTCTGGAGAAACAGC 2936
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 951 ArgSerSerMetGluCysSerProSerAspProThrSerGlyGluThrAl 967

2937 AAGTCAGTCCAACATTAAGTATGCCACTGTTATCAGTAACTCCAGATCAG 2986
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 967 aSerGlnSerAsnIleLysTyrAlaThrValIleSerAsnSerArgSerG 984
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Fig. 9b (continued)

```
2987 GTGGGCTTTACGAACAGAATAAGAATCCCAGATGTCATTTTGATGGATGT 3036
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3037 TTTCTAGCTGAAGACTCCTTGGCTGCAGGTGCTTGCTCAGGTAGCTCCTG 3086
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1001 PheLeuAlaGluAspSerLeuAlaAlaGlyAlaCysSerGlySerSerTr 1017

3087 GGAGCTGGGAAATGAGGCGTTCCTCCTGCTGCCTGACCAGCCTGGCAGCC 3136
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1017 pGluLeuGlyAsnGluAlaPheLeuLeuLeuProAspGlnProGlySerG 1034

3137 AGCCCTGCAAGACCCTCTCCCTTATCTCTTCAGAGGGATTTTCCGAGCCT 3186
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1034 lnProCysLysThrLeuSerLeuIleSerSerGluGlyPheSerGluPro 1050

3187 TCAGATCAGGATGATGCTTTCACAGATGGAGGTAGCCCTGAGCGAGGTCT 3236
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1051 SerAspGlnAspAspAlaPheThrAspGlyGlySerProGluArgGlyLe 1067

3237 CCATTACCTAGGGATAACATCACTGGGCAAAAGAGAAAATGACATTTTTT 3286
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1067 uHisTyrLeuGlyIleThrSerLeuGlyLysArgGluAsnAspIlePheL 1084

3287 TAACAGAAAGTTCCAGACTGATGTGCCATTTCCATACAGCTGATCTGCTC 3336
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1084 euThrGluSerSerArgLeuMetCysHisPheHisThrAlaAspLeuLeu 1100

3337 AGAGGTGTGGGATTTCTTCAGAATACGCCTCCTAATTTAAATGCATTTAT 3386
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1101 ArgGlyValGlyPheLeuGlnAsnThrProProAsnLeuAsnAlaPheIl 1117

3387 CCAGAGTAGCATTAAAGCCATCGTGCCATACGTGCCGCAGTTTCAGATGA 3436
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1117 eGlnSerSerIleLysAlaIleValProTyrValProGlnPheGlnMetT 1134

3437 CTGCTGCCAAGGTGCAAGAGACCACAGAGAACAGCTGT 3474
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Fig. 9b (continued)

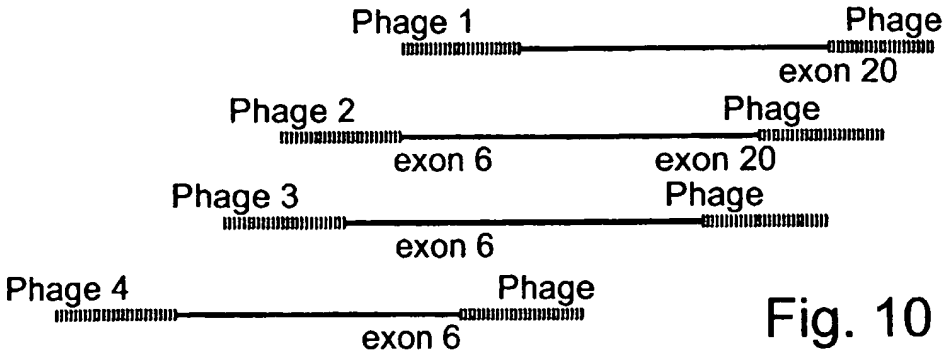

Fig. 10

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<212> PRT

<213> Gallus domesticus

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35 40 45

Ala Ala Gly Val Val Arg Ser Arg Ser Gly Trp Arg Arg Glu His Gly
50 55 60

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Val Ala Glu Thr Asn Pro Ser Leu Leu Met Asp Glu Glu Ser Phe Leu
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Cys Cys Leu Trp Ser Asn Asn Asn Ala Ser Cys Ser Leu Tyr Ser Ala
                85                  90                  95

Asn Met Gln Ala Arg Met Leu Ile Pro Ser Glu Met Ser Ile Ser Ala
            100                 105                 110

Ser Gln Glu Arg Asp Ser Asn Trp Asn Ile Glu Cys Trp Val Glu Gly
        115                 120                 125

Lys Leu Asp Leu Leu Val Cys Ser Leu Gln Phe Pro Lys Phe His Met
    130                 135                 140

Arg Leu Asp Met Lys Val His Leu Leu Tyr Ala Val Ser Glu Leu Ser
145                 150                 155                 160

Leu Gly Asp Thr Ser Thr Ser Thr Leu Lys Arg Thr Ala Leu Ala Ala
                165                 170                 175

Gln Cys Asn Cys Ser Glu Tyr Gly Lys Cys Glu Cys His Val Pro Ser
            180                 185                 190

Pro Arg Leu Asn His Thr Tyr Val Met Trp Leu Lys Thr Gly Ile Gly
        195                 200                 205

Leu Thr Pro Val Trp Ser Pro Leu Met Ser Ala Lys Pro Ile Asp Ile
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Val Lys Pro Asp Leu Pro Leu Asn Val Arg Leu Glu Met Thr Glu Arg
225                 230                 235                 240

Gly Gln Val Lys Ile Cys Trp Ser Glu Pro Val Pro Met Pro Tyr Pro
                245                 250                 255

Leu Arg Cys Glu Val Asn Ile Phe Trp Lys Phe Arg Ser Asn Asp Trp
            260                 265                 270

Gln Gly Val Val Gln Val Ala Leu Asn Thr Ser Leu Asp Ile Asp Asn
        275                 280                 285

Met Leu Leu Asp Ser Ser Ser Phe Ala Gln Val Arg Cys Lys Ser Ile
    290                 295                 300

Cys Val Pro Gly Phe Trp Ser Glu Trp Ser Thr Leu Tyr Asn Leu Asn
305                 310                 315                 320

Gly Glu Val Leu Tyr Phe Pro Thr Lys Ile Leu Thr Ser Val Gly Ser
                325                 330                 335

Asn Val Ser Phe His Cys Ile Tyr Lys Asn Lys Thr Gln Ser Val Ala
            340                 345                 350

Ser Lys Lys Ile Val Trp Trp Leu Asn Leu Ala Glu Glu Ile Pro Glu
        355                 360                 365

Ser Gln Tyr Thr Leu Val Asn Asp Arg Val Ser Lys Val Thr Leu Phe
    370                 375                 380

Asn Leu Lys Ala Thr Lys Pro Arg Gly Ser Phe Phe Tyr Asn Ala Leu
385                 390                 395                 400

Tyr Cys Cys His Gln Asn Arg Glu Cys His His Arg Tyr Ala Glu Leu
                405                 410                 415

Tyr Val Val Asp Val Asn Ile Asn Ile Lys Cys Glu Thr Asp Gly Tyr
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Leu Thr Lys Met Thr Cys Arg Trp Ser Ala Asn Pro Asn Ala Leu Leu
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Leu Gly Ser Ser Leu Gln Leu Lys Tyr His Ser Lys Ile Tyr Cys Ser
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Asn Phe Pro Ser Thr Pro Pro Glu Ser Glu Val Lys Glu Cys His Phe
465                 470                 475                 480

Gln Arg Asn His Ser Tyr Glu Cys Thr Phe Gln Pro Val Phe Leu Leu
                485                 490                 495

Ser Gly His Thr Met Trp Ile Glu Leu Lys His Ser Leu Gly Thr Leu
            500                 505                 510

Glu Ser Ser Pro Thr Cys Val Val Pro Ala Asp Val Val Lys Pro Leu
        515                 520                 525

Pro Pro Ser Asn Ile Lys Ala Glu Ile Thr Arg Asn Asp Gly Leu Leu
    530                 535                 540

Asn Val Ser Trp Thr Asn Pro Val Phe Thr Asn Asp Asp Leu Lys Phe
545                 550                 555                 560

Gln Ile Arg Cys Ala Val Asn Arg Glu Glu Leu Thr Trp Glu Leu Tyr
                565                 570                 575

Glu Val Leu Ser Val Pro Thr Arg Ser Ala Val Ile Glu Val Gln Leu
            580                 585                 590

Cys Val Glu Tyr Ile Val Gln Ile Arg Cys Arg Ala Leu Asp Gly Leu
        595                 600                 605

Gly Tyr Trp Ser Asn Trp Ser Arg Ser Ala Tyr Ala Ala Val Lys Asp
    610                 615                 620

Ile Gln Ala Pro Leu His Gly Pro Glu Phe Trp Arg Thr Val Thr Glu
625                 630                 635                 640

Asp Pro Ala Thr Gly Gln Lys Asn Val Thr Leu Leu Trp Lys Pro Leu
                645                 650                 655

Met Lys Asn His Ser Leu Cys Ser Val Ser Arg Tyr Val Ile Lys His
            660                 665                 670

Gln Thr Ser Glu Asn Thr Ser Trp Ser Glu Tyr Val Asp Asn Gly Thr
        675                 680                 685

Thr Cys Ser Phe Pro Trp Thr Glu Ser Thr His Thr Ile Thr Phe Leu
    690                 695                 700

Ala Val Asn Ser Ile Gly Ala Ser Ser Val Asn Phe Asn Leu Thr Leu
705                 710                 715                 720

Ser Gln Gln Met Ser Thr Val Asn Ala Val Gln Ser Leu Ile Ala Tyr
                725                 730                 735

Pro Val Asn Ser Thr Cys Val Ile Leu Thr Trp Thr Leu Ser Pro Gln
            740                 745                 750

Ile Tyr Val Ile Thr Ser Phe Ile Ile Glu Trp Arg Asn Leu Asn Lys
        755                 760                 765

Glu Glu Glu Met Lys Trp Val Gln Val Pro Pro Asn Ile Ser Lys His
    770                 775                 780

Tyr Ile Tyr Asp His Phe Ile Leu Ile Glu Lys Tyr Arg Phe Ser Leu
785                 790                 795                 800

Tyr Pro Val Phe Ala Ala Gly Val Gly Lys Ser Arg Ala Thr Asp Gln
                805                 810                 815
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Phe Ser Lys Asp Gly Tyr Ala Ser Gln Thr Ser Ser Asn Leu Tyr Met
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Val Leu Pro Ile Val Ile Ser Thr Ser Val Leu Leu Leu Gly Ala Leu
        835                 840                 845

Leu Val Ser His Arg Arg Met Lys Lys Leu Leu Trp Glu Asp Val Pro
    850                 855                 860

Ile Pro Lys Asn Cys Ser Trp Ala Gln Gly Val Asp Phe Gln Gln Pro
865                 870                 875                 880

Glu Thr Phe Glu His Leu Phe Val Lys His Pro Glu Ala Met Ser Phe
                885                 890                 895

Glu Pro Leu Leu Leu Glu Pro Glu Ile Val Leu Glu Asp Ile Ser Val
            900                 905                 910

Thr Lys Ala Leu Glu Gln Glu Asp Thr Gln Asp Phe Leu Ala Leu Asp
        915                 920                 925

Ser Met Phe Thr Lys Pro Glu Asp Ser Glu His Asp Ser Ala Cys Pro
    930                 935                 940

Ser Ser His Phe Ser Gly Arg Ser Ser Met Glu Cys Ser Pro Ser Asp
945                 950                 955                 960

Pro Thr Ser Gly Glu Thr Ala Ser Gln Ser Asn Ile Lys Tyr Ala Thr
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Val Ile Ser Asn Ser Arg Ser Gly Gly Leu Tyr Glu Gln Asn Lys Asn
            980                 985                 990

Pro Arg Cys His Phe Asp Gly Cys  Phe Leu Ala Glu Asp  Ser Leu Ala
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Ala Gly  Ala Cys Ser Gly Ser  Ser Trp Glu Leu Gly  Asn Glu Ala
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Phe Leu  Leu Leu Pro Asp Gln  Pro Gly Ser Gln Pro  Cys Lys Thr
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Leu Ser  Leu Ile Ser Ser Glu  Gly Phe Ser Glu Pro  Ser Asp Gln
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Asp Asp  Ala Phe Thr Asp Gly  Gly Ser Pro Glu Arg  Gly Leu His
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Tyr Leu  Gly Ile Thr Ser Leu  Gly Lys Arg Glu Asn  Asp Ile Phe
    1070                1075                1080

Leu Thr  Glu Ser Ser Arg Leu  Met Cys His Phe His  Thr Ala Asp
    1085                1090                1095

Leu Leu  Arg Gly Val Gly Phe  Leu Gln Asn Thr Pro  Pro Asn Leu
    1100                1105                1110

Asn Ala  Phe Ile Gln Ser Ser  Ile Lys Ala Ile Val  Pro Tyr Val
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Pro Gln  Phe Gln Met Thr Ala  Ala Lys Val Gln Glu  Thr Thr Glu
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